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## (54) AN ARTICLE ADAPTED FOR CONTACT WITH BLOOD, A PROCESS FOR THE PREPARATION THEREOF AS WELL AS USES THEREOF.

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## D scripti n

The present invention relates to the field of heparinization of materials for the purpose of imparting thereto a heparin layer which inhibits in contact with blood the adhesion of thrombocytes and the adsorption of blood proteins. Thus, the article according to the invention is especially suited for applications where there are contacts with blood, e.g. medical applications. It is true that the methodology of heparinizing a material for the above-mentioned purpose is previously known per se, but the present invention relates to a novel, alternative method of adhering the heparin to the substrate, viz. via a special, novel type of a pre-adsorbed layer. In addition to the above-mentioned article the invention relates to a process for the preparation thereof as well as to the use of said article for medical applications.

## BACKGROUND OF THE INVENTION

To accomplish blood compatibility for different materials in contact with blood one of the most important methods has been to heparinize the surface thereof. Thus, the heparin layer on the surface inhibits, as was mentioned above, the adhesion of thrombocytes and the adsorption of blood proteins. Furthermore, the heparin must be enzymatically active in the blood coagulation process, which calls for specific necessities as to molecular conformation and mobility relative to the surface.

Two main principles for the heparinization have previously been utilized. The first one is based on colloidal precipitation, e.g. through complex-formation between amphiphilic amines and heparin. The second one utilizes the possibility of covalently bonding the heparin to the surface. However, these known principles have some limitations which means that there is a continuous research for alternative or improved methods for the heparinization.

## SUMMARY OF THE INVENTION

The present invention relates to an alternative or improved technique for the heparinization of surfaces, which technique eliminates or at least reduces the limitations of the prior art while at the same time imparting thereto, at least for certain applications, additional advantageous properties which have not been obtainable by the previously utilized technique. More specifically we have found that a specific protein, viz. lysozyme, possesses unexpected affinity for heparin and gives an outstanding adhesion to different substrate surfaces. The unexpectedly good results which have been obtained by the protein according to the invention will be described more in detail below, but primarily it can be mentioned that a very good adhesion to metal surfaces has been obtained, which is a material in connection with which previously known methods have shown deficiences.

Lysozyme is a protein that is present in low concentrations in blood. Already therein there is an interesting advantage as in this way the invention is based on the utilization of a substance which is non-foreign to the human organism. In addition thereto another interesting property of lysozyme is its antimicrobial properties which, thus, impart to the novel heparinized surface an antimicrobial activity a security factor in storage and handling.

More specifically the article according to the invention is characterized in that heparin or a heparin-based material is adhered or bonded to the substrate via a layer of lysozyme or a derivative thereof which is pre-adsorbed to said substrate.

As was indicated above the novel technique according to the invention has been shown to work especially well for metal surfaces, in connection with which previously known heparinizing methods have shown limitations. However, the invention is also applicable to other substrates which are chosen per se in accordance with previously known techniques, i.e. primarily such substrates which it has previously been desired to heparinize for the purpose of imparting to the same improved properties in applications where there is a contact with blood. Examples of such materials are polymeric materials and glass. As concerns polymeric materials it should be noted that it has turned out that the invention is especially interesting in connection with polymeric materials of the so called low energy type, which means polymeric materials that are not wetted by water but by organic solvents.

As concerns the term "lysozyme or derivatives thereof" it should be understood that of course the invention is not limited to the use of lysozyme per se but it is also possible to choose any derivative thereof which gives the corresponding or similar properties. Such a choice may for instance depend on a better solubility in the desired solvent for a derivative than for lysozyme per se. As examples of utilizable derivatives there can be mentioned salts, such as the chloride salt. Moreover, the invention is of course intended to cover such cases where the lysozyme has been modified within the molecule at a position or site that does not have any direct connection with the effect of the invention, i.e. a modification that does not change the desired properties according to the present invention.

Nor concerning heparin the requisite is that heparin per se has to be utilized to obtain the desired effect. Thus, the expreesion "heparin-based material" is intended to cover those heparin compounds which give a corresponding or similar effect, reference in this context being made to the prior art which discloses numeral examples of heparin compounds for the purpose referred to. Thus, in this connection the invention does not differ from the prior art.

Those applications for which the articl claimed is

specially well suited are also s lected in accordance with the prior art, which means that this need not be described more here. However, through the fact that certain provements of the properties or additional advantageous properties are obtained by the inv n-tion medical uses or applications will become even more interesting in connection with the invention than according to the prior art.

The process according to the invention is characterized by firstly contacting the substrate with a solution of the lysozyme or the derivative thereof to the formation of a lysozyme layer and then exposing the substrate with its lysozyme layer to a heparin or heparin-based solution so as to adhere or bond the heparin or the heparin-based material to said lysozyme layer.

As is often the case for surfaces which are to be coated, such surfaces have to be comparatively clean to obtain the desired result. This is true also in connection with the present invention, especially in the case where the substrate is a metal. In such a case the surface should be very clean, i.e. be comprised of the metal or the metal oxide. In the ideal case this means that the surface should be cleaned or purified in a so called plasma cleaner and immediately thereafter transferred into distilled water. Alternatively, a consecutive washing in lye, acid and distilled water can be accepted. For a plastic surface, especially a low energetic one, the cleaning preferably means that the material is cleaned in water with a detergent and then an organic solvent. As concerns other substrates in principle those cleaning methods which have previously been utilized in connection therewith are applicable.

After said cleaning of the substrate surface, if required, the substrate i contacted with the lysozyme solution, which is commonly a water solution or an aqueous solution, and distilled water is often preferred relative to a buffer solution. In order to obtain a lysozyme layer the solution should have a concentration of at least 0.1 percent by weight. The upper limit is not especially critical as concerns the desired effect, but generally the concentration should not exceed 10 percent by weight, since otherwise viscosity effects will interfere with the process. An especially preferable range as concerns the concentration of lysozyme or derivative thereof is 0.1-2 percent by weight.

The residence time of said stage of the treatment should be at least 15 minutes, e.g. about 20 minutes, as such a perood is normally required to attain a plateau value for the adsorption of lysozyme. Once said plateau or maximum value has be n attained there is normally no reason to further extend the residence time, which gen rally means that said residence or treatment time is within the range of 15-30 minutes.

After said treatment with lysozyme solution the substrate should be rapidly rinsed in wat r and then directly exposed to a heparin solution or heparin based solution. Thus, it has turned out, especially in connection with metals, that drying should not or must not be performed between the two coating stages, in order to obtain the optimum effect.

Also the solution of heparin or heparin-based compound is preferably water-based. For adsorption reasons its concentration should be above 0.05 percent by weight, especially above 0.1 percent by weight. Nor is in this case the upper limit especially critical, and any additional effect is hardly obtained at a concentration value exceeding about 5 percent by weight. Therefore, a generic range is 0.05-5, especially 0.1-5, percent by weight. However, in many cases said concentration should not even exceed about 2 percent by weight, as otherwise the viscosity will cause interferences. Thus, the specially preferred range is 0.1-2 percent by weight. However, as concerns the heparin treatment in principle all experiences from the prior art can be utilized, i.e. said stage is principally performed per se in accordance with the guide-lines of the prior art in this field.

The exposure time as concerns the heparin solution or the heparin based solution is generally at least 20 minutes, e.g. about 30 minutes, such as 20-45 minutes.

After said exposure to the heparin solution the substrate is suitably rinsed in distilled water, whereupon it is allowed to dry or is dryed after drainage of the excess of solution. By the rinsing in distilled water before said drying the amount of heparin can be reduced to a monomolecular layer. However, for most applications a certain surface excess of dissolved adsorbed heparin is preferred.

For both of the above-mentioned surface treatments it should be noted that they are preferably performed at room temperature. A somewhat raised temperature can be utilized if desired, but generally the temperature should not exceed about 50°C, as otherwise structural changes may appear in the lysozyme.

Finally the invention relates to the use of the above-defined article or of an article prepared by the process defined above, for medical applications where there are contacts with blood. In this connection it should be noted that of course the term "medical applications" should be interpreted in a broad meaning, i.e. the use is not specifically limited to therapeutical treatments only.

## EXAMPLES

The invention will now be further described by means of the following non-limiting xamples. The percentages used th rein relat to p rcentag s by weight unless otherwise specifically stated.

EXEMPLE 1

A commercially available lysozyme from poultry egg white is checked by means of gel electrophoresis to be free from other egg white proteins. The lysozyme is then de-salted by means of dialysis. A solution of 0,5 percent by weight of lysozyme in distilled water is then prepared. Metal cannulae are submersed in a bath of said solution for 20 minutes. Said metal cannulae have been pre-cleaned for 5 minutes in a so called plasma cleanar at an air pressure of 5 torr. They are picked up from the bath, given a shower of distilled water and immediately transferred to a bath consisting of a 0.1% heparin solution in distilled water. After 30 minutes the cannulae are picked up, rapidly given a shower of distilled water and allowed to dry in a sterile chamber at 30°C. In this way metal cannulae having a heparin coating adhered via a pre-adsorbed layer of lysozyme are obtained.

EXAMPLE 2

Catheters of polyethylene are washed in a one percent Triton X100 solution and then in ethanol (96%). Said catheters are submersed in a 0.1% lysozyme solution in distilled water. After about 20 minutes they are passed through a bath of distilled water, whereupon they are transferred to a 0.1% heparin solution in distilled water. After 30 minutes the catheters are washed and are then allowed to dry so as to form articles arcording to the invention.

CLINICAL INVESTIGATION OF HEPARINIZED STEEL TUBES ACCORDING TO THE INVENTION

Several methods have been utilized in order to determine thrombogenicity for artificial materials. A previously utilized method means that steel tubes are inserted into blood vessels and that said steel tubes are incubated in the veesel. During said incubation the coagulation system is incubatad, an adsorption of proteins on the extraneous surface as well as an adhesion of thrombocytes and possible thrombosification of the inserted tube being obtained. From an animal experimental point of view said methodology was found to be a good method especially to study the formation of thrombosis in arteries as well as in veins.

Lately essentially such methods which utilize labeled radio isotopes have been used for studies of thrombogenicity. However, to estimate the thrombogenicity of steel tubes the previously used technique with an intravascular insertion of the steel tube and a determination of the weight differences before and after incubation is the best one for an optimum determination of the thrombogenicity of the material. Steel tubes having a diameter of 4 mm, a length of 25 mm and a thickness of 0.1 mm were heparinized in accordance with Example 1 above.

MATERIALS AND METHODS

Animals: 3 sheep, about 40 kg. Anesthesia: Pentabarbital initially 30 mg/kg, then a continuous infusion with 7.5 mg/min. The sheep intubated, respirator ventilated with 40% of $O_2$, respirator frequency 20/min, volume 10 l/min. Exploration of both carotides, which are opened by a small longitudinal incision, and the 25 mm long steel tube, tapered and polished, is inserted. In carotis on one side there is inserted a heparinized tube and in the other side a non-heparinized tube. Between the different incubations the sides are changed. After pilots test the incubation time was selected to 15 minutes.

RESULTS

25 incubation periods were performed. In all these the thrombus weights were considerably much less on the heparinized tube than on the non-heparinized one (32 + 4 mg as compared to 210 + 10 mg). In addition thereto there were additional thrombus masses in the vessel in seven cases when the steel tube was removed. All these thrombus masses were in non-heparinized tubes (weights 96, 201, 143, 369, 374, 216 and 199 mg).

The statistical calculation when using student's paired t-test gives a t-value of t=9.20, df 25, i.e. a considerably significant reduction of the thrombogenicity.

**Claims**

1. An article adapted for applications where there are contacts with blood, especially medical applications, which article comprises a substrate coated with heparin or a heparin-based material, ***characterized*** in that the heparin or heparin-based material is adhered to the substrate via a layer of lysozyme or a derivative thereof, e.g. a salt, pre-adsorbed to said substrate.

2. An article according to claim 1, ***characterized*** in that the substrate is metal.

3. An article according to claim 1, ***characterized*** in that the substrate is a polymeric material, especially of a so called low energy type, i. e. which is not wetted by water but by organic solvents.

4. A process for the preparation of an article according to any one of claims 1-3, ***characterized*** by first contacting the substrate with a solution, preferably an aqueous one, of lysozyme or a derivative thereof to form a lysozyme layer and then, preferably after a rinsing with water, exposing the substrate with its lysozyme layer to a heparin or heparin-based solution, preferably an aqueous one, to adhere the heparin or heparin-based material to the lysozyme layer.

5. A process according to claim 4, ***characteriz d*** in that the concentration of the lysozyme solution is

0.1-10 percent by weight, preferably 0.1-2 perc nt by weight.

6. A process according to any one of claims 4 and 5, ***haracterized*** in that the heparin solution or heparin-based solution has a c ncentrati n of 0.05-5 p r-cent by weight, preferably 0.1-2 percent by weight.

7. A process according to any one of claims 4 to 6, ***characterized*** in that the residence time for the contact between the substrate and the solution of lysozyme or derivative thereof is at least 15 minutes, especially 15-30 minutes.

8. A process according to any one of claims 4 to 7, ***characterized*** in that the residence time for the exposure with reference to heparin or heparin-based solution is at least 20 minutes, especially 20-45 minutes.

9. A process according to any one of claims 4 to 8, ***characterized*** in that it is performed without any drying operation between the stage of adsorbing lysozyme or derivative thereof and the stage of adhering heparin or heparin-based material.

## Ansprüche

1. Für Anwendungen, in denen Kontakte mit Blut auftreten, insbesondere für medizinische Anwendungen geeigneter Gegenstand, welcher ein mit Heparin oder einem Stoff auf Heparin-Basis beschichtetes Substrat umfaßt, dadurch gekennzeichnet, daß das Heparin oder der Stoff auf Heparin-Basis an das Substrat über eine Schicht aus Lysozym oder einem Derivat davon, beispielsweise einem Salz, gebunden ist, die vorher an dem Substrat adsorbiert wurde.

2. Gegenstand nach Anspruch 1, dadurch gekennzeichnet, daß das Substrat ein Metall ist.

3. Gegenstand nach Anspruch 1, dadurch gekennzeichnet, daß das Substrat ein polymerer Stoff, insbesondere vom sogenannten Nieder-Energie-Typ, ist, d. h. ein solcher Stoff, der von Wasser nicht benetzt wird, jedoch von organischen Lösungsmitteln benetzt wird.

4. Verfahren zur Herstellung eines Gegenstandes nach einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß man

- zuerst das Substrat mit einer Lösung, vorzugsweise einer wäßrigen Lösung, von Lysozym oder einem Derivat davon unter Bildung einer Lysozym-Schicht in Kontakt bringt, und
- danach, vorzugsweise nach Spülen mit Wasser, das Substrat mit seiner Lysozym-Schicht einer Heparin-Lösung oder Lösung auf Heparin-Basis, vorzugsweise einer wäßrigen Lösung, unter Anbindung des Heparins oder d s Stoffes auf Heparin-Basis an die Lysozym-Schicht aussetzt.

5. Verfahren nach Anspruch 4, dadurch gekennzeichnet, daß die Konzentration d r Lysozym-Lösung 0,1 bis 10 Gew.-%, vorzugsweise 0,1 bis 2 Gew.-%, beträgt.

6. Verfahren nach irgendeinem der Ansprüche 4 und 5, dadurch gek nnzeichnet, daß die Heparin-Lösung oder Lösung auf Heparin-Basis eine Konzentration von 0,05 bis 5 Gew.-%, vorzugsweise von 0,1 bis 2 Gew.-%, aufweist.

7. Verfahren nach irgendeinem der Ansprüche 4 bis 6, dadurch gekennzeichnet, daß die Verweilzeit bei Kontakt zwischen dem Substrat und der Lösung von Lysozym oder einem Derivat davon wenigstens 15 Minuten, insbesondere 15 bis 30 Minuten, beträgt.

8. Verfahren nach irgendeinem der Ansprüche 4 bis 7, dadurch gekennzeichnet, daß die Verweilzeit für den Kontakt mit der Heparin-Lösung oder Lösung auf Heparin-Basis wenigstens 20 Minuten, insbesondere 20 bis 45 Minuten, beträgt.

9. Verfahren nach irgendeinem der Ansprüche 4 bis 8, dadurch gekennzeichnet, daß es ohne einen Trocknungsschritt zwischen der Stufe der Adsorption von Lysozym oder einem Derivat davon und der Stufe der Anbindung von Heparin oder dem Stoff auf Heparin-Basis durchgeführt wird.

## Revendications

1. Produit adapté aux applications pour lesquelles il existe des contacts avec le sang, en particulier les applications médicales, lequel produit comprend un substrat revêtu d'héparine ou d'un produit à base d'héparine, caractérisé en ce que l'héparine ou le produit à base d'héparine est amené à adhérer au substrat par l'intermédiaire d'une couche de lysozyme ou d'un dérivé de celui-ci, par exemple un sel, pré-adsorbé sur ledit substrat.

2. Produit selon la revendication 1, caractérisé en ce que le substrat est métallique.

3. Produit selon la revendication 1, caractérisé en ce que le substrat est un matériau polymérique, en particulier du type dit à faible énergie, c'est-à-dire qui n'est pas mouillé par l'eau mais par les solvants organiques.

4. Procédé de préparation d'un produit selon l'une quelconque des revendications 1 à 3, caractérisé par la mise en contact tout d'abord du substrat avec une solution, de préférence une solution aqueuse, de lysozyme ou d'un dérivé de celui-ci pour former une couche de lysozyme puis, de préférenc après un rinçage à l'eau, par l'exposition du substrat avec sa couche de lysozyme à une solution d'héparine ou à base d'héparine, de préférence une solution aqueuse, pour faire adhérer l'héparine ou le produit à base d'héparine sur la couche de lysozyme.

5. Procédé selon la revendication 4, caractérisé en ce que la c ncentration de la solution de lysozyme est de 0,1 à 10% en poids, de préférence de 0,1 à 2% en poids.

6. Procédé selon l'une quelconque des revendications 4 et 5, caractérisé en ce que la solution d'héparine ou la solution à base d'héparine a une concentration de 0,05 à 5% en poids, de préférence de 0,1 à 2% en poids.

7. Procédé selon l'une quelconque des revendications 4 à 6, caractérisé en ce que la durée du contact entre le substrat et la solution de lysozyme ou de dérivé de celui-ci est d'au moins 15 min, en particulier de 15 à 30 min.

8. Procédé selon l'une quelconque des revendications 4 à 7, caractérisé en ce que la durée de l'exposition concernant la solution d'héparine ou à base d'héparine est d'au moins 20 min, en particulier de 20 à 45 min.

9. Procédé selon l'une quelconque des revendications 4 à 8, caractérisé en ce qu'il est mis en oeuvre sans aucune opération de séchage entre l'étape d'adsorption du lysozyme ou d'un dérivé de celui-ci et l'étape d'adhésion de l'héparine ou de produit à base d'héparine.